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(54) Title: USE OF 5'-SUBSTITUTED NUCLEOSIDES TO PREVENT THE DEVELOPMENT OF RESISTANCE DURING TREAMENT WITH CYTOSTATIC AGENTS CONTAINING SAID NUCLEOSIDES

(54) [Title text repeated in German]

(57) Abstract:

The invention involves the use of 5'-substituted nucleosides in combination with at least one cytostatic agent in the production of a medicament which prevents or reduces the development of resistance during treatment with cytostatic agents, as well as a medicament containing BVDU and/or its metabolites.

(57) [Abstract text repeated in German]

THE USE OF 5-SUBSTITUTED NUCLEOSIDES TO REDUCE THE DEVELOPMENT OF RESISTANCE DURING TREATMENT WITH CYTOSTATIC AGENTS CONTAINING THESE NUCLEOSIDES

The appearance of "drug resistance" is the main cause of failure in cancer chemotherapy. Tumors which respond initially to cytostatic agents very often recover after a period of treatment and are then resistant to the action of a variety of anti-neoplastic agents. Although "drug resistance" has been a recognized problem since 1948, when cancer therapy with cytostatic agents began, to date no method has been found to prevent the development of resistance. All highly resistant tumors, which have been investigated so far, have shown amplification (duplication) of a small group of genes. This DNA or gene amplification can be shown to result in increased expression of the gene, which leads to resistance to the drug.

This DNA amplification can be shown to result in increased expression of various genes. Protective proteins, which serve to exclude poisons from the cell, can be over-produced in this way (P-glycoprotein). This effect is named "multi-drug resistance" (MDR).

In addition to MDR, the degree of amplification of certain genes, particularly some oncogenes, correlates with the degree of malignancy. As a result, the chances of surviving gastric cancer with amplification of ERVV2, RASKI, INT3, HST1, MYC and KSRAM (Hirohasi, S, and Sugimura, T. Genetic alterations in human gastric cancer. Cancer cells (Cold Spring Harbor), 3: 49-52, 1991) or ovarian cancer with ERBB2 and MYC (Sasano, H., Garrett, C.T., Wilkinson, D.S., Silverberg, S. Comerford, J., and Hyde, J. Protooncogene amplification and tumor ploidy in human ovarian neoplasm. Hum. Pathol., 21: 382-391, 1990) are very poor. In breast cancer, the amplification of MYC (Borg, A., Baldetorp, B., Fernö, M., Olsson, H., and Sigurdsson, H. C-myc amplification is an independent prognostic factor in postmenopausal breast cancer. Int. J. Cancer, 51:687-691,1992) and co-amplification of INT2 and HST1 (Borg, A., Sigurdsson, H., Clark, G.M., et al., Association of INT2/HST1 coamplification in primary breast cancer with hormone-dependant phenotype and poor prognosis. Br. J. Cancer, 63:136-142,1991) correlate with the prognosis. The amplification of ERBB2 (Descotes, F., Pavy, J.-J., and Adessi, G.L. Human breast cancer: Correlation study between HER-2/neu amplification and prognostic factor in an unselected population. Anticancer Res., 13:119-124,1993)and EGFR (Klijn, J.G.M., Barns, P.M.J.J., Schmitz, P.I.M, and Foekens, J.A. The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review of 5232 patients. Endocr. Rev. 13:3-17,1992) is associated with a poor prognosis. In esophageal carcinoma, co-amplification of HST1 and INT2 correlates with the extent of metastasis (Tsuda, T., Tahara, E., Kajiyama, G., Sakamoto, H., Terada, M., and Sugimura, T. High incidence of coamplification of hst-1 and int-2 genes in human esophageal carcinoma. Cancer Res. 49:5505 5508,1989).

In summary, it has been established that gene amplification induced by chronic treatment with carcinogenic cytostatic agents leads not only to resistance to these agents, but also to over expression of certain oncogenes which control the degree of malignancy.

A range of substances has been described which counteract drug resistance when it develops. These include the anti-cancer actions of the protease inhibitors described in the work of Kennedy (Kennedy, A.R., Prevention of carcinogenesis by protease inhibitors, Cancer Res. 54:1999-2005,1994). These can reduce the levels of carcinogen-induced gene amplification to near normal. Kennedy observed that radiation-induced gene amplification is depressed in a similar way, implying

a connection between the two processes. In addition, protease inhibitors also act as antagonists of (co-recombinant) tumor promotors by triggering transformation *in vitro*. Protease inhibitors are also described as potent anti-promotors in *in vivo* studies (Troll, W., Klassen, A., and Janoff, A. Tumorigenesis in mouse skin: inhibition by synthetic inhibitors of proteases. Science (Washington DC) 169:1211-1213,1970).

The work of Moscow & Cowan (Moscow, I.A., and Cowan, K.H. Multidrug resistance. J. Natl. Cancer Inst. 80: 14-20,1988) has shown that Verapamil counteracts MDR. This "calcium channel antagonist" increases cytotoxicity by enhancing the intracellular accumulation of pharmaceutical agents. It is likely that this is achieved by an action upon P-glycoprotein , or upon other transport proteins. The toxicity of this and similar agents such as Quinidine, for example, prevents them from being used clinically.

Starting from this premise, it is the aim of the current invention to develop a substance, which is effective in preventing or reducing the development of resistance to cytostatic agents, and to produce a corresponding pharmaceutical agent.

This task with respect to a substance is achieved by the attributes described in Claim 1, and with respect to a pharmaceutical agent by the attributes described in Claim 9.

In this invention, it is proposed that simultaneous administration of a 5'-substituted nucleoside together with a cytostatic agent prevents or reduces the development of resistance. Surprisingly, it transpired that the 5'- nucleoside prevents, or at least diminishes, the development of carcinogen-induced gene amplification. This raises the possibility, by co-administration of these nucleosides and cytostatic agents, not only of preventing the development of resistance to the cytostatic agent, but also of influencing the degree of tumor malignancy.

The following are some examples of 5'-nucleosides: 5-(2-bromovinyl-2'-deoxyuridine (BVDU), (E)-5 (2-bromovinyl)-1-β-D-arabinofuranosyluracil, (E)-5-(2-bromovinyl-2'-deoxy-4'-thiouridine. Fig. 2: 5-Iodo-2'-dexycytidine, 5-Iodo-2'-deoxyuridine, 2'-Deoxy-5-trifluoromethyluridine, particularly favorable being BVDU and (E)-5-(2-bromovinyl-)uracil (BVU).

The invention also pertains to pharmaceutical agents aimed at preventing the development of resistance to cytostatic agents, which contain 5' nucleosides. In this case, the pharmaceutical agent

is formulated with a 5- nucleoside content sufficient to produce a blood level of 0.02 to 10 µg/ml. Experiments demonstrated that the optimal range lies between 0.05 and 5 µg/ml.

In this way, the content of the cytostatic agent in the formulation can be kept at the currently familiar level (Oshiro, Y., Piper, C.E., Balwierz, P.S., and Garriot, M.L. (1992) Genotoxic properties of (E)-5-(2-bromovinyl)-2-deoxyuridine (BVDU). Fundamental and applied Toxicology, 18, 491-498). Examples of cytostatic agents include Cyclophosphamide and other alkylating agents, anti-metabolites such as Methotrexate, Alkaloids such as Vinblastine, Antibiotics such as Bleomycin, Cisplatin and other substances. Further examples of cytostatic agents can be found in the "red list 1995" , Editio Cantor Medical & Scientific Press, Aulendorf/Wuertt., 1995.

The carriers, additives and excipients remain the same as those in current usage. The pharmaceutical agent itself can be made as a solid, a liquid or a spray.

The invention is elaborated below using experimental models.

A. Model substances

Experiments on amplification phenomena are performed using a hamster cell line containing a virus (Simian Virus 40) integrated into the genome. The application of both genotoxic substances and various other carcinogenic and tumor-promoting but non-genotoxic substances to this cell line is known to lead to amplification of the SV40 genome in the hamster genome. The method relies upon the hybridization of the probe, labeled SV40-DNA, with hamster cell DNA containing SV40 in amplified copies. The amount of bound probe gives information on the degree of amplification of the integrated viral DNA.

In order to ascertain the degree of amplification, the albumin gene DNA is measured at the same time as the SV40-DNA. In contrast to the SV40-DNA, the albumin gene DNA is not amplified in the cell. The value of the relative SV40-DNA content is derived from the ratio of the signal from the SV40-DNA hybridized probe to that from the albumin gene hybridized probe from the same SV40-transformed embryonic CD631 hamster cells.

The following were used as model substances:

1. Mutagenic and recombinogenic genotoxic carcinogens (positive controls)
   - Triethylenemelamine (TEM)
   - 4-Nitrochinoline 1-Oxide (4-NQO)
2. Non-carcinogens (negative controls) which neither induced mutation nor recombination
   - Acetone
   - Dimethylformide
3. Co-recombinogenic tumor promotors
   - Mezerein
   - 12-O-tetradecanoyl-phorbol-13-acetate (TPA)
   - Chrysarobine
   - Coumarin
3. Recombinogenic, non-genotoxic carcinogens with unknown mechanisms of action
   - Thioacetamide
   - Acetamide
4. Testosterone after metabolism by rat liver microsomes (S9 mix) as well as without S9 mix
   - Testosterone acting
     with S9 mix as an anti-recombinant and
     without S9 mix as a co-recombinant

The actions of the above named model substances were examined alone and in combination with a carcinogen in the gene amplification system.

The results with these model substances are summarized in Figures 1 to 3.

The non-carcinogenic acetone and dimethylformide show no effect.

All other substances, the non-genotoxic carcinogens with uncertain mechanism of action, Thioacetamide and Acetamide, the genotoxic carcinogens TEM and 4-NQO as well as the tumor promoters Mezerein, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), Chrysarobine and Coumarin, when given alone, increase the SV40 gene amplification.

Testosterone with S9 mix reduces the amplifying effect of Methotrexate (MTX), and without S9 mix it increases the amplifying effect of Amino-6-mercaptopurine (AMP).

These results demonstrate a correlation between the production of recombination and of SV40 gene amplification.

B. Restriction of carcinogen-induced gene amplification by (E)-5-(2-bromovinyl-)2'-deoxyuridine (BVDU)

The results are summarized in Figures 4 to 7.

In experiments with yeasts (Figure 4), BVDU demonstrated an anti-recombinant and co-mutagenic action. This action was more obvious in the presence of low concentrations of liver microsomes (S9 mix) than in their absence, as well as being very much more pronounced. Metabolism of BVDU therefore enhances its anti-recombinant effect.

BVDU in clinically relevant doses produces a reduction in AMP-induced gene amplification. The effect starts at a dose of about 0.05 µg/ml and leads in dose-dependant fashion to complete blockade of AMP-induced gene amplification at a dose of 5 µg/ml (Figure 5).

An independent repeat experiment confirmed this result (Figure 6). Furthermore, BVDU alone seems to slightly reduce the degree of spontaneous amplification.

The addition of S9 mix also leads to a reduction in the AMP-induced gene amplification. This occurred, however, at a lower dose range than in the experiments without S9 mix. It seems that possible metabolism of the BVDU results in a further increase in the amplification-limiting effect (Figure 7). This would serve to further increase the relevance of the result.

In summary, it can be stated that BVDU restricts the gene amplification provoked by carcinogens. This raises the possibility of preventing the development of resistance to cytostatic agents by co-administration of BVDU with the cytostatic agent, as well as of reducing the degree of tumor malignancy.

C. Prevention of the development of "multi-drug resistance" (MDR) to cytostatic agents in human and animal tumor cells by simultaneous admission of BVDU.

The human tumor cell line K562-WT (Fig. 8) and the mouse tumor cell line F46-WT (Fig. 9) (WT = Wild Type = sensitive to cytostatic agents = no gene amplification of the MDR gene) were treated with stepwise increasing concentrations of Adriamycin over the course of several weeks. In the course of this treatment, the cells acquired resistance to the treatment. Whereas 20 ng/ml doses of Adriamycin are strongly toxic to non-resistant cells after a treatment time of 4 days, the cells are completely immune to the effects of 20 ng/ml of Adriamycin after prolonged treatment with stepwise increasing doses of the agent (Figures 8 & 9). The development of resistance depends upon amplification of the MDR gene. This is demonstrated using Northern blotting, a technique to demonstrate RNA molecules, i.e. the transcription product of a gene, using the MDR gene as a probe (Fig. 10). Resistant cells show a band whereas non-resistant cells show no band (the situation at the beginning of the treatment).

In parallel experiments, Adriamycin is given together with either 0.5 or 1 µg/ml of BVDU (BVDU is only toxic to human tumor cell lines at concentrations above about 10 µg/ml and to mouse tumor cell lines above about 8 µg/ml (Figs. 8 & 9)). BVDU prevents the development of resistance to Adriamycin. The tumor cells remain sensitive to the cytostatic treatment and die. The action of BVDU is so strong that the treatment has to be interrupted by rest periods (growth without substances), so that the experiment stretches out over 6 to 8 weeks.

BVDU + Adriamycin treatment leads to a substantially weaker amplification of the MDR gene than Adriamycin treatment alone (Fig. 10). The effect of the BVDU treatment is in reality much greater than the attenuation of the band indicates. This is because at the end of the treatment, only those cells, which have developed at least some resistance to the Adriamycin treatment, survive. The cells, which remained non-resistant following the BVDU treatment, have already died off. The truly relevant effect, which cannot be demonstrated by the Northern blot technique, consists, therefore, in the dying-off of non-resistant cells, which is measured in the inactivation curves (Figs. 8 & 9).

Since the development of resistance to cytostatic agents in human tumors also depends upon the amplification of the MDR gene, it should be possible to perform cytostatic therapy at lower doses, and over a more prolonged period, than is currently possible by combining treatment using the desired cytostatic agent together with BVDU. In addition, the prevention of the development of resistance is also of great importance for other applications.

D. Prevention of the development of "multi-drug resistance" (MDR) to cytostatic agents in tumor cells by simultaneous administration of anti-recombinant 5-substituted nucleosides.

It can be seen from Figs. 11 & 12 that the anti-recombinant action is not specific only to BVDU, but is also a property of all 5-substituted nucleosides.

Fig. 11 shows the anti-recombinant effects of (E)-5-(2-bromovinyl)-2-deoxyuridine, (E)-5-(2 bromovinyl)-1-β-D-arabinofuranosyl-uracil and (E)-5-(2-bromovinyl)-2-deoxy-4-thiouridine in *Saccharomyces cerevisiae* MP1 and

Fig. 12 the anti-recombinant effects of 5-Iodo-2-deoxycytidine, 5-Iodo-2-deoxyuridine and 2 deoxy-5-triflurormethyluridine in *Saccharomyces cerevisiae* MP1.

The F4-6-WT mouse tumor cell line (WT = Wild Type = sensitive to cytostatic agents = no gene amplification of the MDR gene) was treated over several weeks with stepwise increasing concentrations of Adriamycin. In the course of this treatment, the cells developed resistance to it. Whereas 20 ng/ml of Adriamycin are toxic to non-resistant cells after a treatment time of 4 days, after long-term treatment with stepwise increasing concentrations the cells became completely insensitive to 20 ng/ml of Adriamycin (Figs. 13 & 14). The development of resistance depends upon amplification of the MDR gene. This can be demonstrated with the Northern blot technique, a procedure which demonstrates the presence of RNA molecules, i.e. the products of gene transcription, when the MDR gene is used as the probe (Fig. 15). Resistant cells show a band, non resistant cells (the situation at the beginning of the treatment) show no band. The level of β-actin mRNA was also analyzed for comparison.

β-actin was used as an internal control for the amount of RNA.

In parallel experiments, Adriamycin was given together with 1 µg/ml of various 5-substituted nucleosides. All six 5-substituted nucleosides prevent the development of resistance to Adriamycin. The tumor cells remain sensitive to the cytostatic agent and die off. The action of the 5-substituted nucleosides was so strong that the treatment had to be interrupted by rest periods (growth without substances), such that the experiment extended over 6 to 8 weeks.

Fig. 15 shows the Northern blot analysis of the RNA:
Expression of the MDR gene in the mouse F4-6-WT tumor cell line. The level of β-actin was also analyzed for comparison. β-actin was used as an internal control for the amount of RNA.

Positive	Adriamycin-resistant F4-6-WT cells
Negative	Adriamycin-sensitive F4-6-WT cells
1	1 µg/ml (E)-5-(2-bromovinyl)-2-deoxyuridine + Adriamycin
2	1 µg/ml (E)-5-(2-bromovinyl)-1-β-D-arabinfuranosyl-uracil + Adriamycin
3	1 µg/ml (e)-5-(2-bromovinyl –2-deoxy-4-thiouridine + Adriamycin

4 1 µg/ml 5-Iodo-2'-deoxycytidine + Adriamycin
5 1 µg/ml 5-Iodo-2'-deoxyuridine + Adriamycin
6 1 µg/ml 2'-deoxy-5-trifluoromethyluridine + Adriamycin
7 1 µg/ml (E)-5-(2-bromovinyl-)-2'-deoxyuridine (BVDU) + Adriamycin

5'-substituted nucleoside + Adriamycin treatment leads to a markedly weaker amplification of the MDR gene than Adriamycin treatment alone (Figure 15). The effect of the treatment is in reality very much greater than the attenuation of the bands indicates. This is because, at the end of the treatment, only those cells which have developed at least some resistance to the Adriamycin treatment survive. Those cells which have remained non-resistant as a result of the BVDU treatment have already died off. The truly relevant effect, which cannot be demonstrated by the Northern blot technique, consists, therefore, in the dying-off of non-resistant cells, which is measured in the inactivation curves (Figs. 13 & 14).

Claims of this Patent

1. The use of 5'-substituted nucleosides in combination with at least one cytostatic agent to produce a pharmaceutical agent which prevents or reduces the development of resistance to treatment with cytostatic agents.

2. Use as in Claim 1, wherein (E)-5-(2-bromovinyl-)-2'-deoxyuridine (BVDU) and/or its metabolites is used as the nucleoside.

3. Use as in Claim 2, wherein (E)-5-(2-bromovinyl-)-uracil (BVU) is used as the metabolite.

4. Use as in Claim 1, wherein (E)-5-(2-bromovinyl)-1-β-D-arabinfuranosyluracil and/or its metabolites is used as the nucleoside.

5. Use as in Claim 1, wherein (E)-5-(2-bromovinyl)-2'-deoxy-4'-thiouridine (BVDU) and/or its metabolites is used as the nucleoside.

6. Use as in Claim 1, wherein 5-Iodo-2'-deoxycytidine and/or its metabolites is used as the nucleoside.

7. Use as in Claim 1, wherein 5-Iodo-2'-deoxyuridine and/or its metabolites is used as the nucleoside.

8. Use as in Claim 1, wherein 2'-Deoxy-5-trifluoromethyluridine and/or its metabolites is used as the nucleoside.

9. Pharmaceutical agents containing 5'-substituted nucleosides meeting at least one of the criteria in Claims 1 to 8, in an amount which results in a blood concentration of 0.02 µg/ml to 10 µg/ml, at least one cytostatic agent and customary binding agents and catalysts.

10. Pharmaceutical agents as defined in Claim 9 wherein the nucleoside is present in an amount which produces a blood concentration in the range 0.05 µg/ml to 5 µg/ml.

11. Pharmaceutical agents as defined in Claims 9 or 10 wherein the cytostatic agent(s) is(are) selected from the alkaloids, alkylating agents, antimetabolites, antibiotics or Cisplatin.

Figures

Fig.1

Aceton	:	Acetone
Dimethylformid	:	Dimethylformide
DNA-Amplifikation	:	DNA amplification
Genotoxische Kanzerogene	:	Genotoxic carcinogens
Kontrolle	:	Control
Negativekontrolle	:	Negative control
Nict-kanzerogene	:	Non-carcinogens
Positivkontrolle	:	Positive control
Relativer DNA-Gehalt	:	Relative DNA content
Zellen des chinesischen Hamsters	:	cells of the Chinese hamster

Fig. 2

Chrysarobin	:	Chrysarobine
Cumarin	:	Coumarin
DNA-Amplifikation	:	DNA amplification
Kontrolle	:	Control
Relativer DNA-Gehalt	:	Relative DNA content
Tumor Promotoren	:	Tumor promotors
Zellen des chinesischen Hamsters	:	cells of the Chinese hamster

Fig. 3

Acetamid	:	Acetamide
DNA-Amplifikation	:	DNA amplification
Kontrolle	:	Control
Nicht-genotoxische Kanzerogene	:	Non-genotoxic carcinogens
Ohne und mit	:	without and with
Relativer DNA-Gehalt	:	Relative DNA content
Testosteron	:	Tesosterone
Thioacetamid	:	Thioacetamide
Zellen des chinesischen Hamsters	:	cells of the Chinese hamster

Fig. 4

Allein	:	alone
Ersatzblatt	:	substitute page
Genmutation	:	gene mutation
Mutanten	:	mutants
Nichtreziproke Rekombinationen	:	non-reciprocal recombinations
Oder	:	or
Regel	:	rule
Reziproke Rekombinationen	:	reciprocal recombinations

Fig. 5

Ersatzblatt	:	substitute page
Kontrolle	:	control
Regel	:	rule
Relativer DNA-Gehalt	:	Relative DNA content

Fig. 6

Allein	:	alone
Ersatzblatt	:	substitute page
Kontrolle	:	control
Regel	:	rule
Relativer DNA-Gehalt	:	Relative DNA content

Fig. 7

Ersatzblatt	:	substitute page
Kontrolle	:	control
Regel	:	rule
Relativer DNA-Gehalt	:	Relative DNA content

Fig. 8

Allein	:	alone
Anzahl	:	number of
Ersatzblatt	:	substitute page
Für 4 Tage	:	for 4 days
Menschlicher	:	human
Regel	:	rule
Wochen Adriamycin	:	weeks of Adriamycin
Zellen	:	cells

Fig. 9

Allein	:	alone
Anzahl	:	number of
Ersatzblatt	:	substitute page
Für 4 Tage	:	for 4 days
Maus	:	mouse
Regel	:	rule
Wochen Adriamycin	:	weeks of Adriamycin
Zellen	:	cells

Fig. 10

Actin-Gen	:	actin gene
Adriamycin allein	:	Adriamycin alone
Als Sonde diente das	:	using as the probe the
Amplifikation	:	amplification
Begin der Behandlung	:	Start of treatment
Der	:	of the
Des	:	of the
Ende der Behandlung mit	:	End of the treatment with
Gens	:	gene
Kontrolle	:	Control
Nachweis	:	proof
MDR-Gen	:	MDR gene
Mit Hilfe des Northern Technik	:	using the Northern blot technique
Zellen	:	cells

Fig. 11

Allein	:	alone
Ersatzblatt	:	substitute page
Nichtreziproke Rekombinationen	:	non-reciprocal recombinations
Regel	:	rule

Fig. 12

Allein	:	alone
Ersatzblatt	:	substitute page
Nichtreziproke Rekombinationen	:	non-reciprocal recombinations
Regel	:	rule

Fig. 13

Allein	:	alone
Anzahl	:	Number of
Ersatzblatt	:	substitute page
Regel	:	rule
Tumorzellen	:	tumor cells
Wochen	:	weeks

Fig. 14

Allein	:	alone
Anzahl	:	Number of
Ersatzblatt	:	substitute page
Regel	:	rule
Tumorzellen	:	tumor cells
Wochen	:	weeks

Fig. 15

Aktin	:	Actin
Ersatzblatt	:	substitute page
Regel	:	rule